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Introduction:

Breast cancer is the second most common malignancy affecting women in the United States. Approximately 182,000 new cases are diagnosed each year.[1] In 1866 Broca was the first to recognize the heritability of breast and ovarian cancer in certain families.[2] In 1990 transmission of breast cancer was genetically linked to a locus on the long arm of chromosome 17.[3] This was followed by the localization of the genes responsible for site-specific hereditary breast cancer and breast/ovarian cancer tandems, BRCA1 and BRCA2.[4,5] These discoveries led to the important quest to determine how these genes function and what if any role they might play in sporadic (non-hereditary) forms of breast cancer.

The types of mutations that have been described in the BRCA genes are consistent with loss of function, a characteristic of tumor suppressor genes. Loss of heterozygosity (LOH) at the BRCA1 locus occurs in approximately 50-70% of sporadic breast and ovarian cancers.[6] LOH at the BRCA2 locus occurs in 30-40% of sporadic breast and ovarian tumors.[7] As with other hereditary tumor suppressor genes (e.g. p53 and APC) it was anticipated that BRCA1 and BRCA2 genes have shown few mutations in non-hereditary breast cancer.[8]

The compelling data that BRCA1 and BRCA2 structural mutations are infrequent in sporadic breast cancer raises questions about the role other cellular events may play in the inactivation of these tumor suppressor genes. One such cellular event which recently has been shown to be significant in colon and renal cell cancer is DNA methylation. The project which I have been working on with this grant investigates the epigenetic modification of DNA methylation in breast cancer. We hope to answer the questions; does this event lead to decreased expression of tumor suppressor genes at the cellular level and does this decreased expression of tumor suppressor genes at the cellular level and does this decreased expression initiate the tumorigenesis cascade.

The factors which determine the accrual of genetic changes during the progression of malignancy are poorly understood. One of the earliest and most consistent molecular changes in neoplasia is not a classic genetic event at all, but rather and alteration in a DNA modification process, cytosine methylation. DNA methylation was first recognized over 40 years ago yet its exact function and importance remain unclear.[9] Loss of expression, wich typically occurs with tumor suppressor genes is a fundamental process underlying the formation and/or progression of many types of cancer. Methylation of the internal cytosine in DNA could potentially participate in such alterations of gene expression. In vitro studies suggest that increased cytosine methylation in promoter regions prevents gene transcription. Increased regional DNA methylation may mark chromosomal changes and gene inactivation events which are central to the genesis and/or progression of human cancers.

The Von Hippel-Lindau gene serves as a model of how hypermethylation plays a role in tumorigenesis. Mutational inactivation and alleleic loss (i.e. loss of heterozygosity) of the VHL gene are causal events for most spontaneous clear-cell renal carcinomas. Hypermethylation of a normally unmethylated CpG island at the 5' end is another potentially important mechanism for VHL gene inactivation in a significant portion of

these cancers. The seminal study of this phenomenon showed that hypermethylation of the VHL gene was found in 19% of the tumors examined.[10] Four of these had lost one copy of VHL while one retained two heavily methylated alleles.[11] Four of the tumors with VHL hypermethylation had no detectable mutations, whereas one had a missense mutation in addition to hypermethylation of the single retained allele.[12] As would be predicted for the consequence of methylation in this 5' CpG island none of the five tumors expressed the VHL gene mutations but no CpG island methylation had expression.[13] In a renal cell culture line, treatment with the demethylating agent 5-aza-2'-deoxycytidine resulted in reexpression of the VHL gene.[14] These findings suggest that aberrant methylation of CpG islands inactivates tumor suppressor genes which prompts tumorigenesis. We propose to investigate whether these criteria apply to breast cancer tumor suppressor genes.

Body:

Activities for the First Year:

I spent the greater part of last summer working out the conditions for the first technical objective of my project proposal which is:

To determine the methylation status of the BRCA1 (breast cancer tumor suppressor gene 1), BRCA2 (breast cancer tumor suppressor gene 2), and RB1 (retinoblastoma tumor suppressor gene 1) tumor suppressor gene promoter regions in breast tumor DNA by restriction endonuclease digestion and Southern blotting.

My tasks for technical objective one from the statement of work are:

Task 1: Months 1-6 Subclone probes for promoter regions of BRCA1, BRCA2 and RB1. Run test Southern blots to determine adequacy of probes.

Task 2: Months 7-10 Extract DNA from breast tumor panel, perform restriction digestions. Run Southern blots, quantitate ratio of uncleaved to cleaved fragments on a phosphorimager.

I began by evaluating the RB1 tumor suppressor gene promoter. The essential promoter region of RB lies 185-206 base pairs upstream of the initiation codon and contains putative binding sites for the transcription factors RBF-1, sp1, ATF and E2F.

These transcription factor binding sites contain numerous CpG islands. There are germ-line mutations affecting these sites which predispose to retinoblastoma. These mutations inhibit the binding of transcription factors and greatly reduce promoter activity. Since RB1 mutations and loss of heterozygosity (LOH) occur in non-hereditary cases of breast cancer I decided to determine if hypermethylation of the RB1 promoter transcription binding sites contributed to LOH. First I used the RB1 promoter sequence to design primers to amplify a 282 base pair PCR product. This product was "gene cleaned" and used as a probe for Southern blotting. The probe contained the RB sequence from coordinates 1679-1961.

Two micrograms of either normal or tumor DNA was digested overnight with PstI or PstI/SmaI and electrophoresed on a 1.5% agarose gel. The cleavage sequence for PstI is CTGC**A G.** The cleavage sequence for Sma I is CC**C GG**G . PstI digestion yields a 2.26 kilobase fragment which is resistant to digestion with SmaI if the internal CpG dinucleotide is methylated. If the CpG dinucleotide is unmethylated the PstI fragment is digested completely in two fragments of 409 and 230 base pairs. After electrophoresis the digested DNA was transferred from the gel to nitrocellulose via capillary action. After transfer the nitrocellulose membrane was probed with the 282 bp fragment.

For the first several experiments I used the standard hybridization procedure that I described in my original project proposal. After obtaining many blots which had significantly intense backgrounds and nonspecific binding I made a few modifications in the procedure. To simplify and standardize the DNA extraction procedure I purchased a commercial kit manufactured by Puregene to replace the "homemade" equilibrated phenol/chloroform reagent that I used in earlier experiments. I added a brief depurination

step by incubating the gel in a solution of low molar hydrochloric acid (0.2N HCl) to facilitate transfer of the DNA from the agarose gel to the nitrocellulose membrane. To make the conditions under which the DNA was transferred from the gel to the nylon membrane less harsh I made the buffer system more alkaline. I switched from a capillary transfer system to a vacuum transfer system (Biorad) to make the transfer procedure faster and more efficient. I adjusted the salt and detergent concentrations of the buffers used during the overnight hybridization step to determine if the background signal on the blots could be quenched. Despite these interventions I have not been able to optimize the transfer conditions to provide clean, easily interpretable, publication quality blots.

During the last two weeks of July, I attended The 38th Annual Short Course in Medical and Experimental Mammalian Genetics. This course is held in Bar Harbor, Maine at the Jackson Laboratory and is jointly sponsored by the laboratory and Johns Hopkins Medical School. The course offered lectures in the morning and evening sessions on a wide variety of topics in genetics. During the afternoon sessions, hands-on workshops were given on topics which were of particular interest to me including techniques for analyzing methylation of tumor suppressor genes.

By September I had tried many modifications of the Southern hybridization protocol without significant improvement in the quality of the blots. Based on information that I obtained from the Bar Harbor course and a thorough review of recently published articles on tumor suppressor gene methylation I decided to use a different experimental approach to address my experimental questions. After reviewing an article in the Proceedings of the National Academy of Sciences by Dr. James G. Herman of the Johns Hopkins Medical School I became convinced that there was a more direct way to detect

methylation in tumor suppressor gene promoters. The procedure is known as methylation-specific polymerase chain reaction (MSPCR).

In his paper Dr. Herman acknowledges many of the difficulties of using Southern blotting for methylation analysis including several that I encountered. In addition he points out that Southern blotting requires large amounts of high molecular weight DNA (5 ug or more) while MSPCR, being a more sensitive technique, can detect methylation in nanogram quantities of DNA. Southern hybridization can detect methylation only if present in great number of alleles while MSPCR can detect very low numbers of methylated alleles. Southern hybridization can only provide information about those CpG sites found within sequences recognized by methylation-sensitive restriction endonucleases. MSPCR allows examination of all CpG dinucleotides. This markedly increases the number of such sites that can be assessed and will allow rapid fine mapping of methylation patterns throughout CpG rich regions. MSPCR also allows the study of paraffin embedded materials which cannot be done with Southern hybridization methods.

Dr. Herman developed a technique which exploits the conversion of DNA with sodium bisulfite and avoids the use of restriction endonucleases. Sodium bisulfite chemically modifies cytosines to uracils. In this reaction all *unmethylated* cytosines are converted to uracils. Those cytosines which are methylated (5-methyl-cytosine) are resistant to this modification and remain as cytosines. This altered DNA can then be amplified by polymerase chain reaction providing detailed information within the amplified region of the methylation status of all CpG sites. Primers are designed to distinguish between methylated and unmethylated DNA in bisulfite modified DNA, taking advantage of the sequence differences resulting from the modification.

Unmodified DNA, or DNA incompletely reacted with bisulfite, can also be distinguished since marked sequence differences exist between unmodified (wild type) DNA and modified DNA.

The procedure is as follows: Genomic DNA is obtained from cell lines, primary tumors and normal tissue via the Puregene DNA extraction kit. DNA (1ug) in a volume of 100 ul of dH2O is denatured by 0.2M NaOH for 10 minutes at 37C. Sodium bisulfite only modifies single strands of DNA so the DNA must be denatured by NaOH prior to modification. The bisulfite reagent will modify very small amounts of DNA, however, if nanogram quantities of human DNA are used 1 ug of salmon sperm should be added as a carrier prior to modification. 30 ul of 10 mM hydroquinone and 520 ul of 3M sodium bisulfite at pH 5, both freshly prepared, are added and mixed and samples are incubated under mineral oil at 50°C for 16 hours. The modified DNA is desalted using the Wizard DNA purification resin according to the manufacturer (Promega) and eluted into 50 ul of water. Modification is completed by 0.3M NaOH (to inactivate the remaining bisulfite) treatment for 5 minutes at room temperature, followed by ethanol precipitation. DNA is resuspended in dH_2O and used immediately or stored at -20°C.

The polymerase chain reaction primers are designed to distinguish between methylated and unmethylated alleles following modification and to distinguish between DNA which has been modified and that which has not been modified. To achieve this objective the primer sequences are chosen for regions containing frequent cytosines (to distinguish unmodified from modified DNA) and CpG dinucleotide pairs near the 3' end of the primers (to provide maximal discrimination in the PCR between methylated and unmethylated DNA). All primers were purchased from Operon.

The PCR mixture contains 10X buffer (100mM Tris-HCL pH 8.3, 500mM KCL), 0.2 uM primers, 25mM MgCl (final concentration 2 mM), 10 mM dNTPs(final concentration 200uM each), and modified or unmodified DNA(< 1ug per reaction) to a final volume of 25ul. Taq gold (2.5 Units/reaction) is used to obviate the need for a "hot start". Amplification is carried out in a Perkin Elmer thermal cycler for 35 cycles. Controls without DNA are performed for each set of reactions. Each PCR product is loaded onto 1% agarose gels, stained with ethidium bromide, and visualized under UV light.

To test the experimental conditions for MSPCR I attempted to reproduce the results from the Herman paper using the p16 allele as a model system (since Dr. Herman's group had already worked out the conditions so nicely) before attempting to use the method on RB1, BRCA2 and BRCA1. The p16 promoter primers were tested on DNA from cancer cell lines and normal tissues for which the methylation status was already known. Lung cancer cell lines H157 (which has a methylated p16 allele) and H209 (which has an unmethylated p16 allele) were ordered from ATCC and grown in culture. DNA was extracted and modified with sodium bisulfite. The H157 DNA amplified only with the methylated primers. The H209 DNA amplified only with the unmethylated primers. DNA not treated with bisulfite failed to amplify with either the methylated or unmethylated primers. The unmodified DNA readily amplified with primers for the sequence before modification (wild type primers).

After my success with the p16 allele I began applying the MSPCR protocol to analyze the RB promoter. From the promoter sequence I designed primers which would amplify wild type, methylated and unmethylated sequences of the Rb promoter. Thus far I have

been able to get the wild type primers to amplify DNA from the serum leukocytes of a breast cancer patient (Please see Figure 3 in appendix). I have had difficulty getting the methylated and unmethylated primers to work. At this point it is still unclear if the procedure is not working because of incomplete modification with the sodium bisulfite or if the PCR conditions are not optimal for product amplification. I am continuing to work on primer design since it is critical that the primers not be too long and that the sequence end with a "cg clamp" at the 3' end to ensure proper annealing. I am also working on the modification conditions since significant amounts of DNA can be lost during the procedure due to degradation from the low pH (5) and from the purification on the Wizard prep columns. Lastly I am working on the amplification conditions, particularly annealing temperatures.

Another difficulty that I must address is finding appropriate controls for the experiment. I am consulting with Dr. Thaddeus Dryja from the Massachusetts Eye and Ear Institute to obtain samples of retinoblastoma tumors which have documented methylated and unmethylated alleles. I plan to use these tumor DNAs as positive controls to facilitate my analysis. One of the most difficult aspects of performing MSP with "new" genes (like the breast cancer genes) is finding the appropriate control DNAs. These controls are easily obtained (from the commercial vendor, Oncor) for the p16, p15 and VHL tumor suppressor genes because their methylation status has been extensively studied in labs which specialize in methylation analysis. For tumor suppressor genes whose methylation status has not been studied, the controls must be developed by each individual researcher.

In addition to working out the conditions for studying the methylation status of the RB1 gene I have been performing a similar exercise for BRCA2. The BRCA2 promoter region has binding sites for the transcription factors ELF (nucleotides 2138-2147) and USF (nucleotides 2178-2182). These sites contain CpG dinucleotides and are excellent candidates for sites which are hypermethylated. Hypermethylation of either of these sites would prevent the appropriate transcription factor from binding which would lead to decreased expression/LOH.

The ELF site contains a CCGG sequence which is the recognition sequence for the restriction endonucleases MspI and HpaII. As I described in my proposal these restriction endonucleases are isoschizomers. MspI cuts at the sequence C**CG**G regardless of the methylation status of the internal cytosine. HpaII recognizes the same sequence but is inhibited by a methylated internal cytosine. I performed the restriction digest on 10 ug of DNA extracted from normal leukocytes, breast cancer cell lines and excised breast tumors. I performed the electrophoresis and nitrocellulose transfer as described earlier. For the hybridization step I used a ^{32}P-radiolabeled probed created from a fragment of DNA which contained a sequence from the promoter region of the BRCA2 gene which was 324bp in length. Cleavage of DNA by MspI gives bands of 143bp and 181 bp. HpaII digestion of DNA when inhibited by methylation gives a 324bp band. I did not have any greater success with the BRCA2 Southern blots than I had with the RB1 blots. I attempted the same optimization interventions with no improvement.

Next I tried to apply the principles of methylation specific PCR to the BRCA2 promoter region. I designed primers which would amplify both the ELF and USF binding sites in unmodified DNA, unmethylated modified DNA and methylated modified

DNA. Thus far I have only been able to amplify the unmodified DNA. I am approaching this problem the same way I approached the RB1 problem; by assessing primer design, sodium bisulfite modifications conditions and PCR amplification conditions.

One method that I am going to utilize to determine the completeness of the sodium bisulfite modification is genomic sequencing. I have been reading about a method developed by Myohanen, Wahlfors and Janne which employs automated fluorescent sequencing to analyze modified DNA. The method requires DNA to be modified as described previously. Modified DNA is amplified by polymerase chain reaction with "outer primers" which cover a region of 500-800 bp. The resulting PCR product is amplified with nested "inner primers" chosen to cover a region of less than 500 bp. The product from the second PCR reaction is sequenced using fluorescent tagged dideoxynucleotides (Perkin-Elmer Applied Biosystems) and an automated sequencer. By comparing the sequence to modified to unmodified DNA I will be able to tell if the sodium bisulfite modification actually worked by changing unmethylated cytosines to uracils. I have designed the outer and inner primers and I am currently doing the PCR amplification reactions.

The methylation project has consumed most of my research time. Lengthy incubation times, trouble-shooting and waiting for reagents to be ordered or developed has allowed me time to think about another project which interests me. I have spent some of my spare research time working on it. I am not abandoning the methylation project and I will continue to try to get it to work. I would like to describe the other project that I have been working on during the past year since I may pursue it on a full-time basis if the methylation project results do not improve in the next twelve months.

Rapamycin is a macrolide antibiotic which has antifungal and immunosuppressive activities. It is currently being used in clinical trials as an immunosuppressive agent in solid organ transplant recipients. Rapamycin and FK506, another macrolide immunosuppressive agent currently in clinical use, share structural homology. Both drugs are able to bind to FKBP12 (FK506 Binding Protein). When complexed with FKBP12, FK506 can inhibit T cell receptor signaling by calcineurin. When complexed with FKBP12, rapamycin can inhibit the IL2 signaling pathway. Thus rapamycin and FK506 are reciprocal antagonists. When an excess of rapamycin is added to T cells inhibited with FK506 the T cell receptor pathway is restored but the IL2 pathway is inhibited and vice versa. The targets of the rapamycin/FKBP12 complex are the TOR1 and TOR2 (Target of Rapamycin) proteins. These are large 250kD proteins with 67% homology to each other. They also share a C terminal domain with similarity to both protein and lipid (PI3/PI4) kinases. TOR2 is an essential protein and together with TOR1 is required for progression from G1 to S phase in the cell cycle.

In mammalian cells rapamycin causes a dramatic increase in the proportion of G1 phase cells. Rapamycin also disrupts a ras-independent signal transduction pathway required to activate p70S6K. p70S6K is a kinase which phosphorylates the ribosomal protein S6 which when blocked by phosphorylation would inhibit protein translation. It also blocks the downregulation of the p27Kip1 protein. The p27Kip1 protein is an inhibitor of the cdk2/cyclin E which is required for transition of cells from G1 to S phase. Thus the available G1 cyclin-cdk complexes remain saturated with Kip1 and are unable to execute critical regulation functions like phosphorylating the Rb protein which are required for the cell to advance through the cell cycle.

I wanted to study the effect of rapamycin of breast cancer cells to see if it would have the same effect on the tumor cells as it has on T cells. First I did a cytotoxicity assay to determine if the cells were sensitive to the drug and at what concentration I could induce cell death. I performed the assay with XTT, a yellow tetrazolium salt, which is reduced in living metabolically active cells to an orange formazan product produced by mitochondrial dehydrogenases. From the assay I determined that a reasonable starting concentration would be 1 ng/ml.(Please see Figure 4 in appendix)

Next I wanted to determine if that concentration of rapamycin could induce G1 arrest in MCF7 cells. I starved MCF7 cells for forty-eight hours, induced cell cycling with estrogen and subsequently added rapamycin. After twenty-four hours I harvested the cells and stained them with BRDU (and detected the BRDU with a monoclonal antibody)and propidium iodide for fluorescent activated cell sorting. I was able to induce G1 arrest with the rapamycin.(Please see Figure 5 in appendix).

The next question I wanted to answer was is this effect occurring at the promoter level for estrogen responsive genes or is it occurring in an estrogen responsive cell signaling pathway? I obtained a plasmid which contained three estrogen response elements and a luciferase reporter from a collaborator at Duke, Dr. Donald McDonald. I performed a transient transfection of this plasmid into MCF7 cells (with SuperFect transfection reagent by Qiagen) and induced them to proliferate with estrogen. I then added rapamycin to see if it would cause a decrease in promoter activity. I found that the rapamycin did cause significant diminution of the signal.(Please see Figure 6 in appendix). This led me to conclude that the effect of rapamycin in MCF7 cells is

occurring at the level of the promoter not necessarily at the level of the estrogen signaling cascade.

Another question is what, if any, effect rapamycin will have on tumors in vivo. I would like to collaborate with Dr. Mark Dewhirst of the department of pathology at Duke on this project. We have plans to inject MCF7 cells into mice in a dorsal subcutaneous location and stimulate tumor growth with estrogen since MCF7 cells are estrogen responsive. I would then administer rapamycin to the mice over the course of seven to ten days. At the end of that time period the tumor would be weighed and measured and compared to tumors from mice which received no rapamycin to see if the size of the tumor decreased. I am currently discussing the logistics of the experiment with Dr. Dewhirst and planning toxicity experiments to determine if rapamycin can be administered to mice safely.

Conclusions:

With regard to the first technical objective of my original proposal and statement of work I have developed probes to perform Southern blot analysis of the methylation status of the RB1 promoter and the BRCA2 promoter. Thus far I have not been able to produce Southern blots which are clear enough to reveal the methylation status of the two promoters. I have attempted to answer the question by using another technique called methylation specific PCR which allows selective amplification of methylated and unmethylated CpG islands in DNA which has been modified with sodium bisulfite. Sodium bisulfite changes unmethylated cytosines in CpG islands to uracils. Thus far I have not been able to amplify the DNA with the specifically designed primers.

Once I have determined the methylation status of a select group of tumors for RB1, BRCA2 and BRCA1 I will pursue the other objectives from my original proposal one of which is to compare the levels of the mRNA for RB1, BRCA2 and BRCA1 in see if the levels of DNA methyltransferase are increased in tumors which are methylated.

I will continue to pursue the effect of rapamycin in breast cancer cells by investigating what effect it might have on estrogen signaling pathways and by investigating the effect it might have on transplanted tumors in mice.

References:

1. Herman, JG, Graff, JR, et. al. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. PNAS 93: 9821-9826.